ONR 001AC

Identification of Specific Protein Markers that Correlate with Initiation and Progression of Microbially Influenced Corrosion

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Geo-Microbial Technologies, Inc.

1992

Office of Naval Research

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Final Report
November 30, 1992

Contract Number N00014-92-C-0063

Prepared for
Office of Naval Research
Department of the Navy

800 N. Quincy Street Code: 1413:NRJ
Arlington, Virginia 22217-5000

Abstract

The feasibility of using sodium dodecyl sulfate polyacrylamide gel electrophoresis and 2-dimensional gel electrophoresis as techniques for identification of specific protein markers that correlate with initiation and progression of microbially influenced corrosion was demonstrated during Phase I. Results showed that proteins can be detected in extracts of oil pipeline pigging solids and in concentrated water samples from locations where corrosion coupon data show evidence of corrosion. Different protein banding patterns were observed for samples collected from different locations. Samples collected from the same location at different times also showed a slight change in banding pattern. These results are significant because changes in protein banding patterns can be indicative of changes in microbial populations or of metabolic activities associated with initiation of microbially influenced corrosion (MIC). Capability to detect proteins in water samples is especially significant because identification of markers in the water will provide the earliest indication of MIC. The need for optimizing sample preparation procedures to remove interfering components and conditions for 2- D electrophoresis to obtain maximum resolution was discussed. Recommendations for future research in Phase II that would lead to development of a simple and economical diagnostic procedure for early identification of MIC were made.

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A. Summary

Microbially influenced corrosion (MIC) is increasingly being recognized as a significant contributing factor to the degradation and failure of structural materials. Estimates of economic losses due to corrosion are nearly $ 200 billion a year in the United States, although it is not known how much is due to MIC. Current methods for detection of MIC are expensive and time consuming. Frequently, they involve a battery of chemical, electrochemical, metallurgical, and microbiological tests that include cultural tests. Consequently, damage has already occurred before MIC is identified. Thus, there is a need for development of a simple, rapid, and economical method for early detection of MIC so that corrective measures can be instituted before damage occurs. Availability of such a method would significantly lower economic losses due to corrosion.

Specific characteristics and metabolic activities of microorganisms are a function of the constituent enzymes and proteins of any particular species. In addition, enzymes and proteins that perform the same function in microorganisms have shared as well as unique characteristics. Some are expressed in response to environmental conditions. Therefore, identification of specific protein markers correlating with initiation and progression of MIC would be more truly representative of the consortium of microorganisms involved in MIC, including their metabolic interrelationships. Protein markers identified will be the basis for development of a simple and economical diagnostic procedure for early detection of MIC

In Phase I of this project, we proposed to determine the feasibility of using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 2-dimensional (2-D) gel electrophoresis as techniques for identification of specific protein markers correlating with initiation and progression of MIC. SDS-PAGE is a technique that separates proteins according to their molecular weights after treatment with SDS and 2-mercaptoethanol. High resolution 2-D gel electrophoresis separates proteins according to their isoelectric points in one dimension and according to their molecular weights in the second dimension. This technique has the capability of resolving complex protein mixtures into their individual components. Data obtained showed that proteins can be detected by SDS-PAGE in extracts of oil pipeline pigging solids and in concentrated water samples from injection wells and water tanks where corrosion coupon data show evidence of corrosion. In addition, different protein banding patterns were observed for samples collected from different locations. Samples collected from the same location at different times also showed a slight change in banding pattern. These results are significant because changes in protein banding patterns can be indicative of changes in microbial populations or of metabolic activities associated with

initiation of microbially influenced corrosion (MIC). Resolution of the proteins detected into their individual components by 2-D gel electrophoresis was not totally achieved due to interference from high molecular weight contaminants present in the pigging solid extracts. These contaminants caused streaking, diminished resolution, and masked portions of the gel preventing visualization of some protein spots. Evidence was obtained suggesting that these contaminants could be high molecular weight polysaccharides. Alternatively, the proteins themselves could be highly glycosylated. Nevertheless, one sample yielded a fairly good 2-D protein map. These results demonstrate the feasibility of using these techniques for identification of specific protein markers correlating with stage of corrosion. The problems encountered due to interfering contaminants are solvable. The sample preparation procedures can be modified to remove interfering components and conditions for 2- D electrophoresis can be optimized to obtain maximum resolution. Recommendations for future research in Phase II that would lead to development of a simple and economical diagnostic procedure for early identification of MIC were discussed.

B. Introduction

Microbially influenced corrosion (MIC) is increasingly being recognized as a significant contributing factor to the degradation and failure of structural materials. Annual economic losses due to corrosion have been estimated to be nearly $ 200 billion in the United States (1). It is not known how much is due to MIC. However, it has been suggested that microorganisms are responsible for approximately 75 % of corrosion in productive oil wells in the U. S. Current methods for detection of MIC are often laborious, time consuming, and expensive. Frequently, they involve a battery of chemical, electrochemical, and microbiological tests that include culturing specific types of microorganisms that have been implicated in MIC. Consequently, significant damage has already occurred before MIC is detected. Thus, there is a need for a simple and economical diagnostic procedure that can be incorporated in a monitoring program to facilitate early identification of MIC. Detection of MIC prior to initiation of pitting damage will allow timely institution of corrective measures and significantly lower economic losses due to corrosion.

The goal of Phase I research is to demonstrate the feasibility of SDS-PAGE and 2-D gel electrophoresis in identification of specific protein markers that correlate with initiation and progression of MIC. The variety of microorganisms and the mechanisms whereby they cause and enhance corrosion have been reviewed (2, 3). The roles of slime producing, filamentous, acetogenic and sulfate reducing bacteria in biofilm formation and MIC are subjects of active research investigations (2 - 12). These have contributed much to our understanding of the underlying complex mechanisms involved in MIC. The metabolic activities of microorganisms

are functions of the constituent enzymes and proteins of any particular species. In addition, enzymes or proteins that perform the same function in different microorganisms have shared as well as unique characteristics. Changes in metabolic activities or states of microorganisms are a result of differential expression of proteins in response to environmental conditions. Proteins can be detected in viable cells as well as non-viable cells and their degradation products. Therefore, identification of protein markers correlating with initiation and progression of MIC would be more truly representative of the consortium of microorganisms involved in the sequence of events that lead to corrosion than would cultural tests. Protein markers identified will form the basis for development of a simple and economical diagnostic procedure for early identification of MIC.

C. Methods

Development of a screening method for detection of proteins in oil pipeline fluids and solids

Screening of samples for detectable protein was done by 1-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D SDS-PAGE). This technique separates proteins according to their molecular weights following treatment with SDS and 2-mercaptoethanol at 100 °C. Initial efforts concentrated on the development of a suitable sample preparation protocol for oil pipeline pigging solids prior to electrophoresis. The sample preparation procedure that resulted in good 1-D SDS gels is described as follows:

Approximately 2 grams wet weight of each sample were weighed out into a 25 ml glass Corex centrifuge tube with a teflon-lined screw cap. Hydrocarbon extraction was performed once with 15 ml of n-hexane with shaking in a wrist action shaker at room temperature for one hour. Hexane was removed following centrifugation at 8,000 x G for 15 minutes. Hydrocarbon extraction was repeated with 15 ml toluene under the same conditions as extraction with n-hexane. The solids were then washed with 10 ml of a 1:1 mixture of methanol and acetone with shaking at room temperature for 15 minutes. The methanol-acetone wash was removed following centrifugation at 8,000 x G for 15 minutes. The pellet was allowed to dry by evaporation of any residual methanol-acetone under a fume hood at room temperature. The solids were then extracted with 5 ml of 10% SDS in 0.05 M 2-[N-cyclohexylamino]-ethanesulfonic acid (CHES), pH 9.5 with shaking at room temperature overnight. The SDS extract was transferred to a 15 ml Corex centrifuge tube following centrifugation at 8,500 x G for 20 minutes and set aside. A second extraction with 3 ml of 10% SDS in 0.05 M CHES pH 9.5 was performed with shaking at room temperature for one hour. The SDS extracts were combined and any carried over particulates were removed by centrifugation at 8,500 x G. A 4 ml aliquot of the combined SDS

extracts was transferred to a 15 ml Corex centrifuge tube and 2 ml of 60% (w/v) trichloroacetic acid (TCA) were added. The tube was kept in ice for one hour to allow precipitation of proteins in the extract. The tubes were centrifuged at 8,500 x G for 30 minutes. The pellets were washed with ice cold 95 % ethanol. The washed pellets were redissolved in a minimum volume of 10% SDS in 0.05 M CHES pH 9.5. These were the samples used for SDS-PAGE.

SDS-PAGE

The procedure followed for SDS-PAGE was essentially that of Laemmli (13) using BioRad's Mini Protean II electrophoresis system. Samples were diluted 1:1 with sample buffer and heated in a boiling water bath for 10 minutes. After cooling, 25 µl were applied to the gel using a Hamilton syringe. A 12 % acrylamide separation gel was used. The sample and gel solutions used and the electrophoresis buffer composition are shown in Table 1. Electrophoresis was carried out at a constant voltage of 200 V for 40 minutes. Duplicate gels were usually run so that two methods of staining could be used.

Protein Staining

Two methods of staining were used for visualization of protein bands following electrophoresis. Staining with Coomasie Brilliant Blue (CBB) G-250 was performed according to the method of Neuhoff et. al. (14). This method utilizes the colloidal properties of 0.1 % (w/v) CBB in 2% (w/v) phosphoric acid, 10% (w/v) ammonium sulfate, 20% (v/v) methanol which produces sensitive staining of proteins on a clear background. After electrophoresis, the gel is fixed in 12% (w/v) TCA for a minimum of one hour with gentle shaking. Fixing the gel overnight results in less background and more defined bands. The fixed gel is immersed in the staining solution for 2 to 4 hours with gentle shaking. After staining, the gel is briefly washed with 25 % (v/v) methanol. For prolonged storage, the gel was transferred to a solution of 20% (w/v) ammonium sulfate in water.

Silver staining was carried out using BioRad's Silver Stain Plus Kit. The recommended procedure was followed with minor modifications. Extensive fixation (overnight) with two changes of the fixative solution and longer rinsing times in water were required to obtain good staining of protein bands with a minimum of background. In addition, staining time was reduced from 20 minutes to 5 minutes. Staining for 20 minutes resulted in a totally black gel.

Protein Assay

The method chosen for quantitative determination of protein in samples used for SDS-PAGE was the method of Smith, et. al. (15) using bicinchoninic acid (BCA) purchased from Pierce Chemical Co. This method showed the least sensitivity to SDS. Presence of up to 1% SDS in the sample did not interfere with the assay. However, only samples precipitated by TCA gave reliable results which were comparable to band intensities obtained on SDS-PAGE. Attempts to measure protein in SDS extracts prior to precipitation with TCA grossly overestimated the protein concentration in the extracts. Similar results were obtained using the Bradford (16) method which was more sensitive to SDS interference.

High resolution 2-dimensional (2-D) gel electrophoresis

The sample preparation method developed for 1-D SDS-PAGE was modified to make the samples more suitable for 2-D PAGE. Initial results with samples prepared as described above showed considerable interference from unknown components which caused streaking and resulted in poor resolution. We assumed that this could be due to presence of ions including excess SDS which are known to interfere in isoelectric focusing. Furthermore, the initial sample preparation protocol developed included precipitation of the proteins with 20% TCA as a means of concentrating the dilute extracts as well as remove interfering components that grossly overestimated protein concentration when measured by the BCA method. Hence, desalting techniques such as ion exchange chromatography on AG 11 A8 (BioRad) resin which binds small ions, ultrafiltration using Ultrafree-CL (Millipore) and dialysis through membranes with molecular weight (MW) cut-offs of approximately 10, 000 were performed prior to 2-D electrophoresis.

The extraction procedure was also modified following the observation that treatment of the pigging solid samples with 6 N H_2SO_4 prior to hydrocarbon extraction resulted in release of microorganisms and oil from the particulate matrix. Precipitation of the SDS extracts with TCA was eliminated and concentration was achieved using Aquacide II to absorb most of the water from the samples contained in dialysis bags. The modified sample preparation procedure is described as follows:

Approximately 2 grams (wet weight) solid sample with varying amounts of water, oil and corrosion products were weighed out into 25 ml teflon-lined screw-capped glass centrifuge tubes. 10 ml 6 N H_2SO_4 were added and shaken vigorously. The suspension was extracted with 10 ml n-hexane with vigorous shaking in a mechanical shaker at room temperature for 30 min twice. Separation of the phases was achieved by centrifugation at 10,000 x G for 15 min at 4 °C. The hydrocarbon phase were removed and discarded. Residual n-hexane was removed by evaporation in a fume hood. The

aqueous phase was centrifuged at 1,000 x G for 5 min to remove large particulates (P-1). The turbid supernatant (containing microorganisms and debris) was transferred to a teflon centrifuge tube and spun at 15,000 x G for 30 min. The supernatant was removed and the pellet (P-2), containing microorganisms and debris was saved. The first pellet (P-1) was extracted with 15 ml toluene with vigorous shaking for 30 min at RT. The toluene phase was removed following centrifugation at 10,000 x G for 15 min. The toluene-extracted pellet was washed with 10 ml 50% methanol-50% acetone (v/v). Residual methanol-acetone was removed by evaporation in a fume hood. The pellet (P-1) was resuspended in 10 ml deionized water and centrifuged at 1,000 x G for 5 min. The turbid supernatant containing more released microorganisms and debris was added to the pelleted microorganisms in the first extraction (P-2), resuspended in a total volume of 20 ml deionized water and centrifuged at 15,000 x G for 30 min. The pellet was washed once more with 20 ml deionized water to remove any residual acid, then resuspended in 5% SDS in 0.05 M CHES, pH 9.5. The suspension was shaken at room temperature in a wrist action shaker overnight. The suspension was centrifuged at 15,000 x G and the supernatant removed and saved. The pellet was resuspended in 1/2 the volume of the first SDS extraction and then placed in a boiling water bath for 30 minutes. After cooling the boiled suspension was centrifuged and the supernatant taken and added to the first SDS extract. The combined SDS extract was transferred to a dialysis bag with a MW cut-off of 6,000 to 8,000 and dialyzed exhaustively versus deionized water at 4° C. Following dialysis the extract was concentrated by covering the dialysis bag with Aquacide II at 4° C overnight. Aquacide II is a high molecular weight (500,000) carboxymethylcellulose powder with very high water-absorbing capacity. The dialyzed and concentrated SDS extract was used for 2-D gel electrophoresis and carbohydrate analysis.

Two dimensional gel electrophoresis in our laboratory was performed according to the method of O' Farrell (17) using BioRad's mini 2-D cell. The samples were diluted 1:1 with first dimension sample buffer and 25 µl (containing from 1 - 10 µg protein) were loaded on the tube gels. The sample, gel, and electrode solutions used for the first dimension are shown in Table 2. Isoelectric focusing was carried out in capillary tubes of 1. 0 mm inner diameter using 1.3 % pH 5 - 7 (Sigma) and 0.7 % pH 3 - 10 (BioRad) ampholytes for 3,000 volt-hrs. Electrophoresis in the second dimension was carried out in a 12 % acrylamide slab gel (1. 0 mm thick), after equilibration of the tube gel in SDS-reducing buffer for 10 min, for 40 min at a constant voltage of 200 volts. The 2-D gels were stained with silver as described above. The stained gels were dried between sheets of cellophane paper with the acid edge to the left.

Two dimensional electrophoresis on standard sized gel (13.5 x 15.5 cm) performed by Kendrick Labs, Inc. (Madison, WI) was also carried out by the method of O' Farrell (17). Isoelectric focusing in the first dimension was performed in glass tubes of 2. 0 mm inner diameter using 2% pH 4 - 8 ampholines (BDH from Hoefer Scientific Instruments) for 9600 volt-hrs. Electrophoresis in the second dimension was carried out in a 10 % acrylamide slab gel (0.75 mm thick) after equilibration for 10 min in sample buffer (10 % glycerol, 50 mM dithiothreitol, 2.3 % SDS and 0.0625 M tris, pH 6.8) for 4 hrs at 12.5 mA/gel. The slab gels were fixed in 10 % acetic acid/50 % methanol overnight and silver stained (18). The stained gels were dried between sheets of cellophane paper with the acid edge to the left.

Analysis of water samples

Water samples were concentrated 100 fold prior to electrophoresis. 50 ml of the water sample were placed in a dialysis bag with a MW cut-off of 6,000 to 8,000, covered with Aquacide II powder and left in the refrigerator overnight. The almost dried sample in the dialysis bag was allowed to rehydrate in deionized water for approximately 1 hour and the sample recovered. Samples were brought to a final volume of 0.5 ml with deionized water. If sample volume was greater than 0.5 ml, volume was reduced by ultrafiltration using Ultrafree-CL filter (Millipore) with a MW cut-off of 10,000.

Total carbohydrate analysis

Total carbohydrates was measured by the phenol-sulfuric acid reaction (19).

D. Results and Discussion

The initial sample preparation procedure which included a TCA precipitation step gave reasonably good results on 1-D-SDS-PAGE. A number of pigging solids (Table 3) were analyzed according to the above procedure. The protein concentrations of the TCA-precipitated extracts which were used for electrophoresis are shown in Table 4. Photographs of the resulting gels stained with either CBB G-250 or silver are shown in Figures 1 - 4. These results show that different protein banding patterns can be observed in samples obtained from different locations. For example, in Figs. 1 and 2, lane 5, a sample collected from the paddle of a gas flotation cell that removes oil and solids from produced water, shows a markedly different pattern from lanes 2, 3, and 4, which were pigging solids from drill site (DS) 11 (a manifold building for gathering well line flows into common lines), pigging solids from the line connecting IMF2 (an intermediate manifold building for

seawater) to DS 11, and pigging solids from the produced water injection line of DS 4, respectively. Lanes 2 and 3 in Fig. 1, a gel stained with CBB G-250, appear to be identical and very similar to lane 4. However, subtle differences in the relative intensities of the protein bands in each sample as compared to Fig. 1, can be observed in Fig. 2, a duplicate gel stained with silver.

Figures 3 and 4 are also duplicate gels stained with CBB G-250 and silver, respectively. Again different banding patterns can be observed from samples collected from different locations. The most interesting information that can be obtained from these gels are manifested in lanes 3, 7, and 8. These samples were pigging solids from the produced water injection line of DS 13 but were collected on different dates. Lanes 7 and 8 (samples collected on 7/29/90 and 11/25/90, respectively) show a major band (identified by an arrow in Fig. 3) which is not present in lane 3 (sample collected on 4/10/90). This band stains yellow in the duplicate silver stained gel (Fig. 4). It is possible that this protein band consists of lipo- and/or sialo-glycoproteins which have been shown to stain yellow with silver (20). This result is significant because the appearance of an additional protein band could mean a change in microbial population or a change in metabolic activities which could signal initiation of MIC.

Following the successful development of a screening method for detection of proteins in oil pipeline fluids, we proceeded to develop methods for 2-D gel electrophoresis. We have found that the sample preparation protocol developed for 1-dimensional SDS-PAGE was not totally adequate for 2-D gel electrophoresis. Initial experiments showed considerable interference from unknown components which caused streaking and resulted in poor resolution . We initially assumed that this could be due to presence of ions including excess SDS which are known to interfere in isoelectric focusing. Furthermore, the initial sample preparation protocol developed included precipitation of the proteins with 20% TCA as a means of concentrating the dilute extracts as well as remove interfering components that grossly overestimated protein concentration when measured by the bicinchoninic acid (BCA) method. Desalting techniques such as ion exchange chromatography on AG 11 A8 (BioRad) resin which binds small ions, ultrafiltration and dialysis through membranes with molecular weight (MW) cut-offs of approximately 10, 000 were performed prior to electrophoresis. These techniques did not result in appreciable improvements in the 2-D gels obtained (Figs. 5-8). Since these techniques removed only small MW contaminants (<10,000), we concluded that the interfering components are high molecular weight compounds. Two possible high molecular weight contaminants are nucleic acids and polysaccharides. To determine whether the interfering contaminants are nucleic acids, two samples were treated with deoxyribonuclease (DNase) and ribonuclease (RNase) in ice prior to 2-D gel electrophoresis. No appreciable improvement in the results were obtained. Analysis of the samples for total carbohydrate content by the phenol reaction

(19), revealed relatively high carbohydrate concentrations (Table 5). Hence, we now believe that a major portion of the interfering components are high molecular weight polysaccharides or the proteins themselves could be highly glycosylated. Occurrence of densely glycosylated glycoproteins containing as much or more mass as carbohydrate than as polypeptide is known (21). Dense glycosylation results in heterogeneous structures and polydisperse molecular weights which can cause streaking or smearing on gel electrophoresis. Despite these problems, we were able to obtain a reasonably good protein map (on a standard sized gel, 13.5 x 15.5 cm) for one sample with discernible spots although some streaking still masked some areas of the gel (Fig. 9). This result demonstrates the feasibility of identifying specific protein markers correlating with stage of corrosion by SDS-PAGE and 2-D gel electrophoresis.

In order to conclusively identify specific markers, sufficient numbers of samples need to be analyzed so that statistically significant correlations can be made. In addition, appropriate samples for correlation with corrosion data need to be collected. Field data on corrosion status of oil pipelines are limited. The only information available was pitting damage measured ultrasonically. It was not deemed appropriate to use corrosion coupon data for correlation with pigging solid samples because it has been observed in the past that there is a lack of correlation between such data and actual corrosion status of the pipes (personal communication with corrosion engineer of oil company). Of the pigging solid samples analyzed from four locations where protein bands where detected in their SDS extracts, only one showed a slight pitting damage. This apparent lack of correlation can be attributed to the fact that the pigging samples were a conglomerate of the solids collected from a vast area, while pitting damage is localized. In addition, these locations were being treated continuously with corrosion inhibitors, although no biocide treatment was in effect at the time the samples were collected. The corrosion inhibitors, which were quaternary amines, could have effected protection. Thus, appropriate samples need to be collected from localized areas of varying degrees of corrosion.

Analysis of water samples yielded a more positive correlation with corrosion coupon information obtainable. The amount of protein present in the water samples were so low that it was necessary to concentrate them at least 100 fold to be detectable by silver staining. However, it can be seen that corrosion rate of coupons could be roughly correlated with the number and intensity of bands (estimated visually) detected on silver stained SDS-PAGE gels (Table 6, Figs. 10, 11). Only one (Injection well D, Table 6), out of 10 samples analyzed, did not correlate with coupon data. This finding is significant because the ultimate goal is the development of a simple diagnostic tool for early detection of MIC. Water samples are much easier to collect and analyze. If proteins are detectable in appropriately concentrated water samples, it is reasonable to suggest that any proteins identified as markers of MIC would likewise be detectable in the water. Thus, we believe

we have demonstrated the feasibility of using 1-D and 2-D gel electrophoresis as methods for identifying specific protein markers correlating with stage of corrosion.

E. Conclusions

Data obtained in Phase I demonstrated the feasibility of using 1-D-SDS-PAGE and 2 -D gel electrophoresis as techniques for identifying specific protein markers correlating with initiation and progression of MIC. Results showed that SDS-PAGE can detect proteins in extracts of oil pipeline pigging solids and in appropriately concentrated water samples from locations where coupon data show evidence of corrosion. However, sample preparation procedures and conditions for analysis by 2-D gel electrophoresis need to be optimized . High molecular weight contaminants, probably polysaccharides caused interference due to streaking and masked protein spots toward the acidic end of the gel. Alternatively, the proteins themselves could be densely glycosylated. These two possibilities are very likely considering the fact that extracellular polysaccharides are often involved in bacterial adhesion and biofilm formation (11). These problems are solvable as there are a number of methods available for separating carbohydrates from proteins or special techniques for purification of glycoproteins. Methods for deglycosylation of glycoproteins are also available and the reagents required are commercially available. Therefore, with additional research and development in Phase II, the identification of specific protein markers correlating with initiation and progression of MIC can be achieved. A simple and economical diagnostic procedure based on these markers will be developed for early identification of MIC. Availability of such a procedure will significantly reduce economic losses by the federal government and industry due to corrosion.

F. Recommendations

Additional research must be done to realize the the full potential of the technical approach for identification of MIC proposed in Phase I. This will entail optimizing the sample preparation protocol as well as conditions for 2-D electrophoresis to obtain maximum resolution of proteins in the samples. In addition sufficient numbers of solid and water samples need to be analyzed to obtain statistically significant correlation. Appropriate samples need to be collected for correlation with corrosion information obtainable. The most appropriate samples would probably be the solid deposits from localized areas representing the stages of biofilm formation and development of pitting corrosion. These stages are divided into 1) bacterial adhesion, 2) colony formation, EPS production of adhering bacteria, initiation of pitting corrosion, 3) mature biofilm and enlargement of pit (11, 12). Actual field samples of these type would not be easy to come by but are obtainable. Pigging

solids as well as produced water samples should also be analyzed for comparison with the samples collected from localized areas of varying stages of corrosion. Since the ultimate goal is the development of a simple and economical diagnostic kit for identification of MIC, detection of the markers in the water or pigging solids would provide an early indication of MIC.

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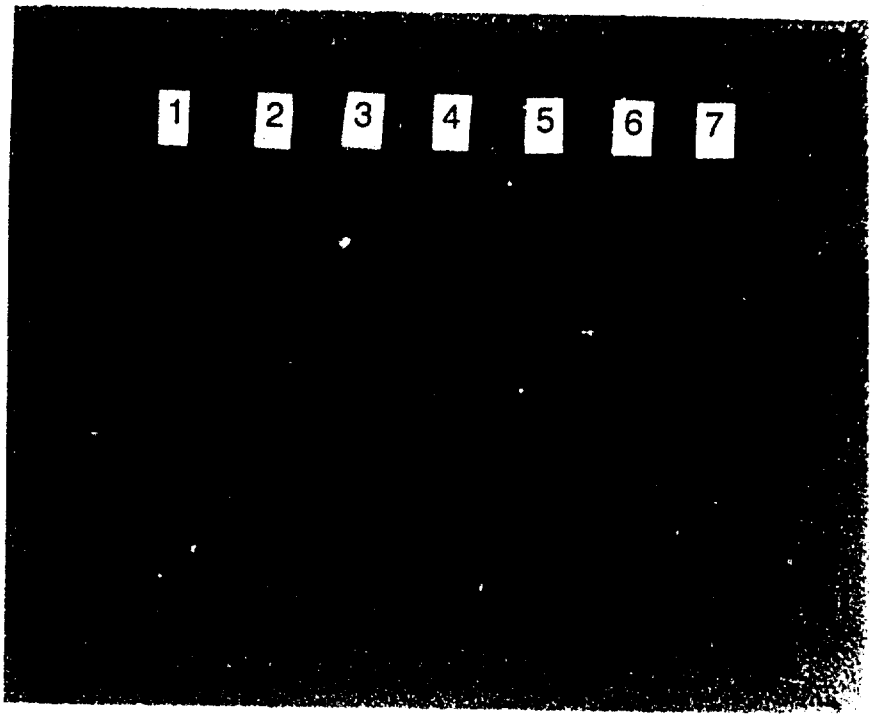

Figure 1. SDS-PAGE of solid samples stained with CBB G-250. Lane 1, MW standards (97.4, 66.2, 45.0, 31.0, 21. 5, 14.4 Kd); lane 2, sample # 1, DS 11 pig solids, 4/3/90 (5.2 µg); lane 3, sample # 2, IMF2-DS 11 pig solids, 4/3/90 (8.8µg); lane 4, sample # 14, DS 4 PWI pig solids, 5/1/90 (5.9 µg); lane 5, sample # 99, FS 2 GFC1 paddle solids, 8/2/90 (14 µg); lane 7, E. coli cell lysate (66 µg).

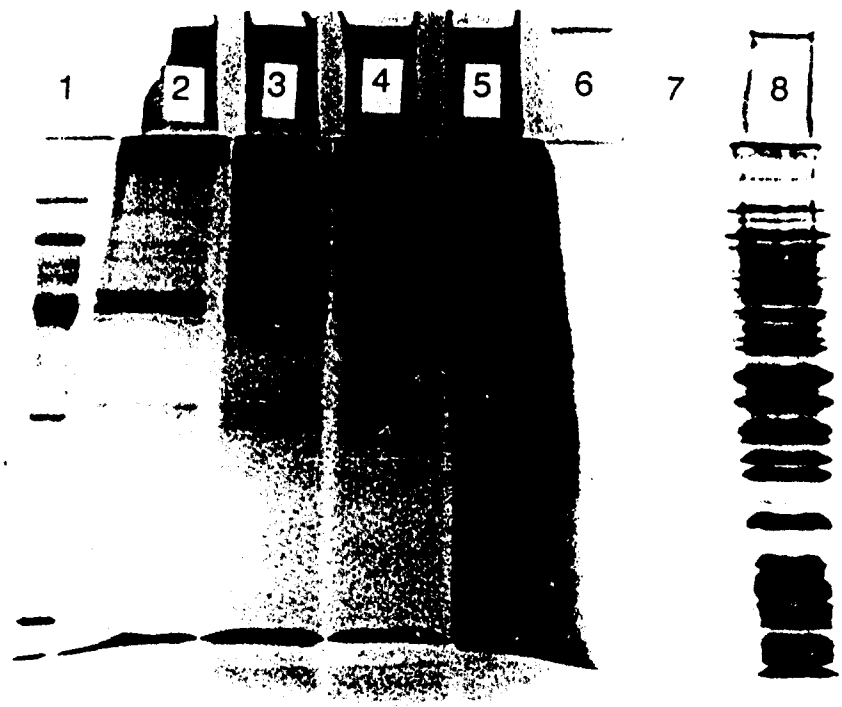

Figure 2. SDS-PAGE of solid samples stained with silver. Lanes 1-5, same samples as in Fig. 1. E. coli lysate was loaded in lane 8.

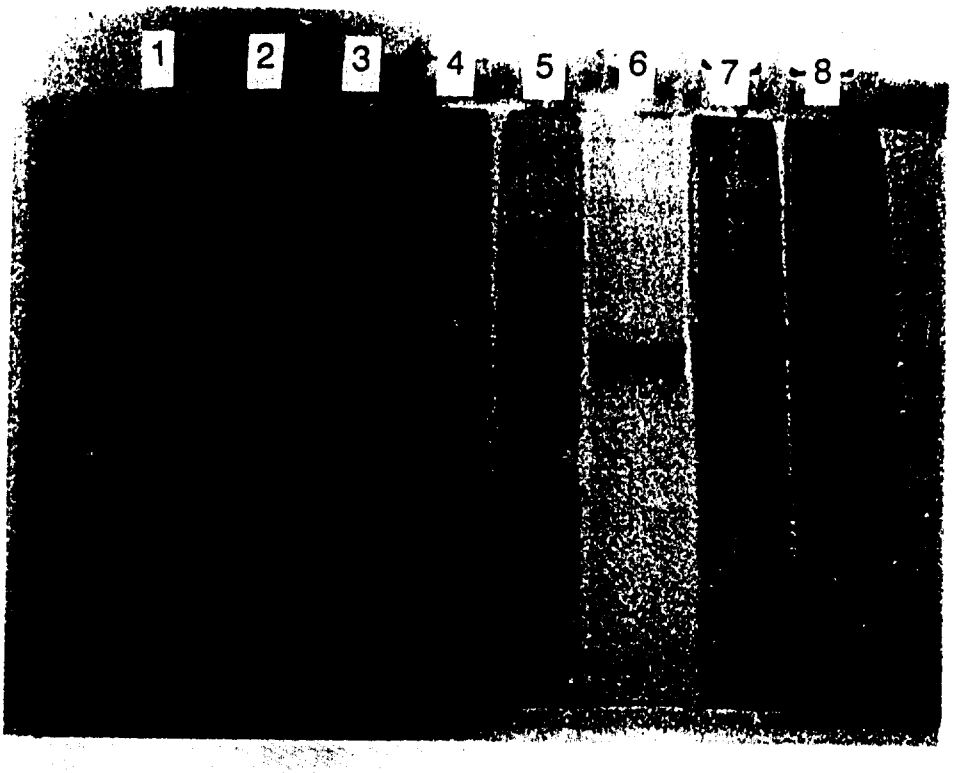

Figure 3. SDS-PAGE of solid samples stained with CBB G-250. Lane 1, MW standards (an old preparation was used, showing deterioration of the protein bands); lane 2, sample # 3, IMF2-DS 11 pig receiver solids, 4/7/90 (4.5 µg); lane 3, sample # 7, DS 13 PWI pig solids, 4/10/90 (22 µg); lane 4, sample # 13, DS 4 PWI pig solids, 5/1/90 (5.2 µg); lane 5, sample # 15, DS 4 PWI pig trap solids, 5/1/90 (5.9 µg); lane 6, sample # 73, DS 9 PWI pig trap solids, 6/9/90 (2.5 µg); lane 7, sample # 103, DS 13 PWI pig trap solids, 7/19/90 (18 µg); lane 8, sample # 160, DS 13 PWI pig trap solids, 11/25/90 (18 µg); (note arrow on additional band in lanes 7 and 8 compared to sample in lane 3).

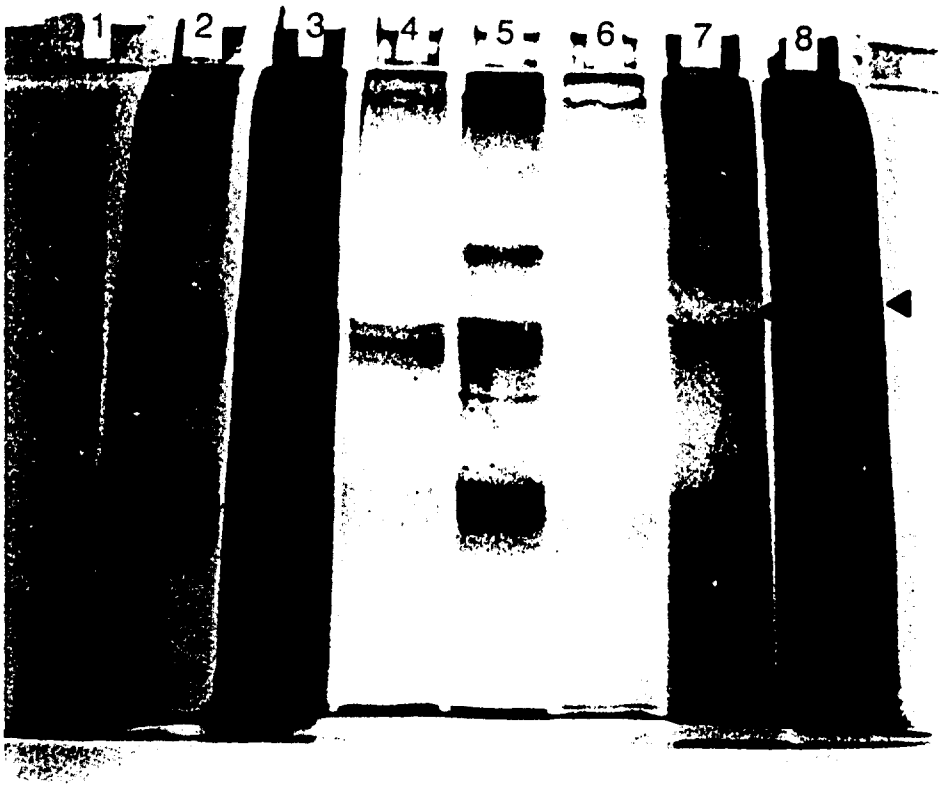

Figure 4. SDS-PAGE of solid samples stained with silver. The same samples in each lane as in Fig. 3 (note arrow on additional band in lanes 7 and 8, compared to sample in lane 3).

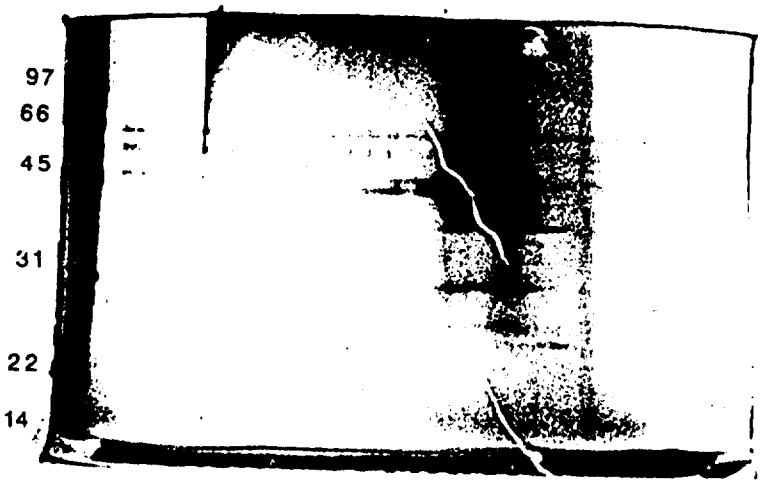

Figure 5. 2-D-PAGE on a 12 % acrylamide mini gel of sample 13 (1 µg protein) after ultrafiltration. Isoelectric focusing in the first dimension was done in 1.3 % pH 5-7 and 0.7 % pH 3-10 ampholytes. Bands on the left-most side are MW standards, phosphorylase b (97 Kd), bovine serum albumin (66 Kd), ovalbumin (45 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21.5 Kd), and lysozyme (14 Kd). The acidic end is toward the left and the basic end is toward the right. The gel was stained with silver.

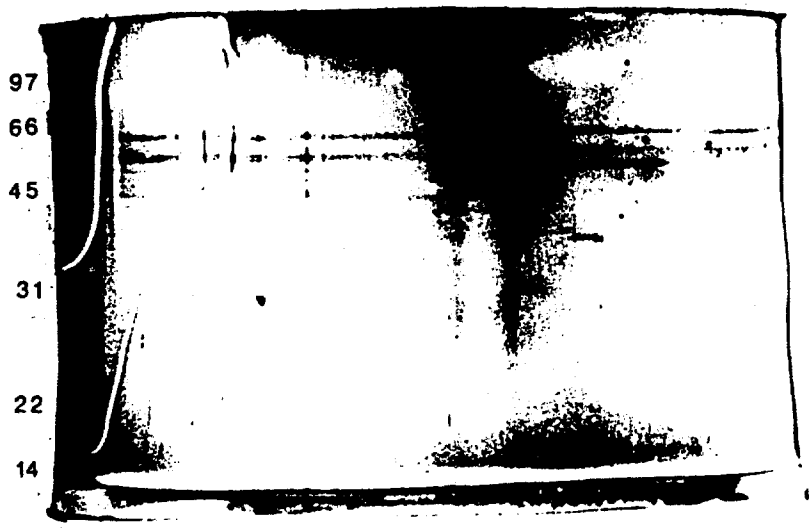

Figure 6. 2-D-PAGE on a 12 % acrylamide mini gel of sample 160 (2 µg protein) after ultrafiltration. Isoelectric focusing in the first dimension was done in 1.3 % pH 5-7 and 0.7 % pH 3-10 ampholytes. Bands on the left-most side are MW standards, phosphorylase b (97 Kd), bovine serum albumin (66 Kd), ovalbumin (45 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21.5 Kd), and lysozyme (14 Kd). The acidic end is toward the left and the basic end is toward the right. The gel was stained with silver.

Figure 7. 2-D-PAGE on a 12 % acrylamide mini gel of sample 13 (1.6 µg protein) after passage through an ion exchange column and ultrafiltration. Isoelectric focusing in the first dimension was done in 1.3 % pH 5-7 and 0.7 % pH 3-10 ampholytes. Bands on the left-most side are MW standards, phosphorylase b (97 Kd), bovine serum albumin (66 Kd), ovalbumin (45 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21.5 Kd), and lysozyme (14 Kd). The acidic end is toward the left and the basic end is toward the right. The gel was stained with silver.

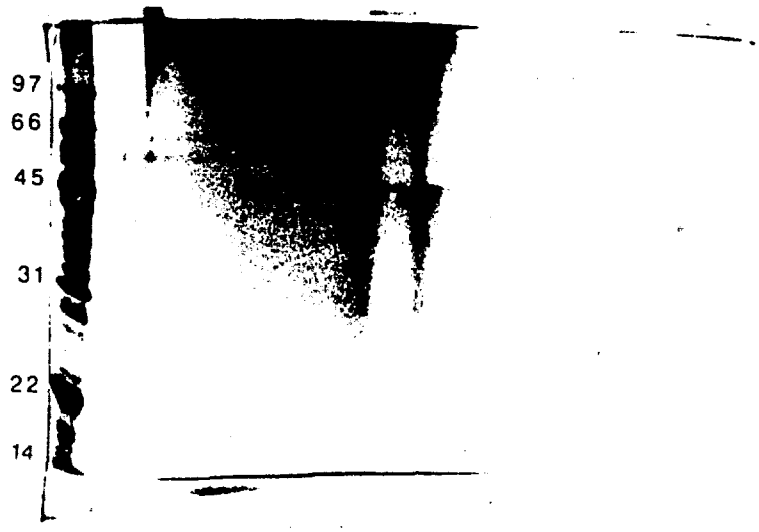

Figure 8. 2-D-PAGE on a 12 % acrylamide mini gel of sample 166 (35 µg protein) after passage through an ion exchange column and ultrafiltration. Isoelectric focusing in the first dimension was done in 1.3 % pH 5-7 and 0.7 % pH 3-10 ampholytes. Bands on the left-most side are MW standards phosphorylase b (97 Kd), bovine serum albumin (66 Kd), ovalbumin (45 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21.5 Kd), and lysozyme (14 Kd). The acidic end is toward the left and the basic end is toward the right. The gel was stained with silver.

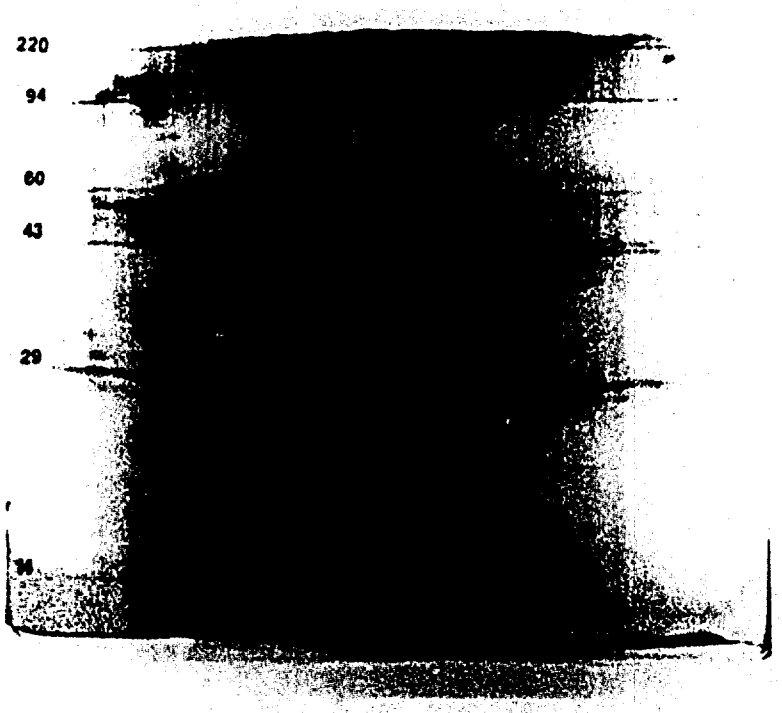

Figure 9. 2-D-PAGE of sample 160 (30 μg protein) on a standard sized (13.5 x 15.5 cm) 10% acrylamide gel. The lines across the gel are due to MW standards, myosin (220,000), phosphorylase a (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000). Isoelectric focusing in the first dimension was carried out in 2% pH 4-8 ampholytes. The acidic side is toward the left and the basic side is toward the right. This run was performed by Kendrick Labs, Inc. (Madison, Wisconsin).

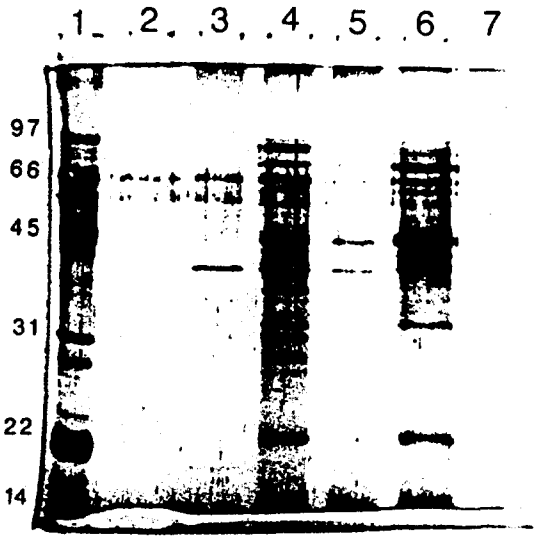

Figure 10. SDS-PAGE of produced water samples (concentrated 100x) stained with silver. Lane 1, MW standards, phosphorylase b (97 Kd), bovine serum albumin (66 Kd), ovalbumin (45 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21.5 Kd), and lysozyme (14 Kd); lane 3, inlet produced water tank (PWT) -1; lane 4, injection well D; lane 5, inlet, PWT-3; lane 6, injection well E; lane 7, inlet PWT-2. 25 µl of each sample were loaded on the gel.

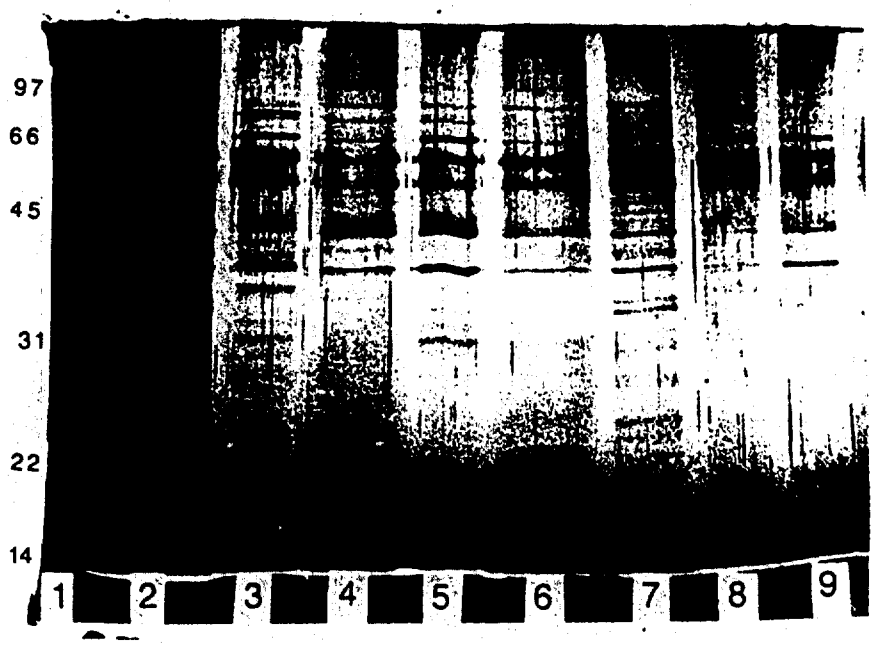

Figure 11. SDS-PAGE of produced water samples (concentrated 100x) stained with silver. Lane 1, MW standards, phosphorylase b (97 Kd), bovine serum albumin (66 Kd), ovalbumin (45 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21.5 Kd), and lysozyme (14 Kd); lane 2, inlet PWT-1; lane 3, injection well D; lane 4, inlet PWT-3; lane 5, injection well E; lane 6 inlet PWT-2. 25 µl of each sample were loaded on the gel. Lanes 7-9 are repeats of Lanes 2-4, respectively, loading 10 µl of each sample.

Table 1

Separation Gel, Sample, and Electrophoresis Buffer Solutions

12% Separation Gel

Stock Solution	Volume
Distilled water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.50 ml
10% (w/v) SDS	100 µl
Acrylamide/Bis (30%)	4.00 ml
10% (w/v) ammonium persulfate	50 µl
Total volume	10.0 ml

Sample Buffer (SDS reducing buffer)

Stock Solution	Volume
Distilled water	3.2 ml
0.5 M Tris-HCl, pH6.8	1.0 ml
Glycerol	1.6 ml
10% (w/v) SDS	1.6 ml
ß-mercaptoethanol	0.4 ml
0.1% (w/v) Bromophenol Blue	0.2 ml
Total volume	8.0 ml

Electrophoresis Buffer Composition, pH 8.3

Tris base	3.0 g/L
Glycine	14.4 g/L
SDS	1.0 g/L

Table 2

Composition of Gel and Sample buffers Used for First Dimension Isoelectric Focusing

First dimension gel monomer solution

9.2 M urea
4 % acrylamide (total monomer)
2 % NP-40
1.6 % pH 5-7 ampholyte
0.4 % pH 3-10 ampholyte
0.01 % ammonium persulfate
0.1 % TEMED

First dimension sample buffer

9.5 M urea
2 % NP-40
5 % 2-mercaptoethanol
1.7 % pH 5-7 ampholyte
0.3 % pH 3-10 ampholyte

First dimension sample overlay buffer

9 M urea
0.8 % pH 5-7 ampholyte
0.2 % pH 3-10 ampholyte
0.0025 % bromophenol blue

Cathode buffer: 20 mM NaOH

Anode buffer: 10 mM H_3PO_4

Table 3

List of Pigging Solid Samples Analyzed by SDS-PAGE

Sample #	Date Collected	Sampling Location or Area
1	4/3/90, 9:43	Drill Site (DS) 11 pig solids
2	4/3/90, 9:42	IMF2-DS 11 pig solids
3	4/7/90	IMF2-DS 11 pig receiver solids
13	5/1/90	DS 4 produced water injection (PWI) pig solids
14	5/1/90, 11:11	DS 4 PWI pig solids
15	5/1/90	DS 4 PWI pig trap solids
7	4/10/90	DS 13 PWI pig solids
103	7/29/90	DS 13 PWI pig trap solids
160	11/25/90	DS 13 PWI pig trap solids
73	6/9/90	DS 9 PWI pig trap solids
99	8/2/90	FS2 (Flow Station 2) GFC1 paddle solids

FS 2 GFC1 paddle, the paddle off of a gas flotation cell inside Flow Station 2

Table 4

Protein Concentrations of Extracts from Solid Samples Used for SDS-PAGE

Sample #	Date Collected	Sampling Location or Area	Protein (mg/ml)
1	4/3/90	DS 11 pig solids	0.42
2	4/3/90	IMF2-DS 11 pig solids	0.70
3	4/7/90	IMF2-DS 11 pig receiver solids	0.36
13	5/1/90	DS 4 PWI pig solids	0.42
14	5/1/90	DS 4 PWI pig solids	0.47
15	5/1/90	DS 4 PWI pig trap solids	0.47
7	4/10/90	DS 13 PWI pig solids	1.73
103	7/29/90	DS 13 PWI pig trap solids	1.42
160	11/25/90	DS 13 PWI pig trap solids	1.45
73	6/9/90	DS 9 PWI pig trap solids	0.20
99	8/2/90	FS 2 GFC1 paddle solids	1.12

Table 5

Total Carbohydrate and Protein Content of SDS extracts of Pigging Solids

Sample #	Date Collected	Carbohydrates mg/ml	Protein mg/ml	Carbohydrate/Protein Ratio
2	4/3/90	3.54	1.12	3.2
7	4/10/90	5.07	7.72	0.6
13	5/1/90	4.82	2.96	1.6
14	5/1/90	4.82	2.96	1.6
73	6/9/90	4.53	5.59	0.8
103	7/29/90	6.22	4.25	1.5
160	8/2/90	5.62	8.67	0.6

Table 6

Correlation of 1-D-SDS-PAGE Results of Water Samples with Corrosion Coupon Data

SAMPLE: DESCRIPTION	DATE COLLECTED	CORROSION COUPON DATA							Number and Intensity of Bands on Gels
			CORROSION RATE (MPY)						
		EXPOSURE (Days)	OVERALL "1"		BODY PIT "2"		END PIT "3"		
			Value	Rating	Value	Rating	Value	Rating	
Seawater, Injection Well A	5/25/92	193	0.10	a	2	a	2	a	ND
Produced Water, Injection Well B	5/11/92	188	5.80	d	14	c	12	c	"+"
Produced Water, Injection Well C	5/22/92	191	1.30	b	17	c	13	c	"+"
Produced Water, Injection Well D	6/8/92	100	0.20	a	0	a	7	b	"+++"
Produced Water, Injection Well E	6/8/92	191	9.30	d	52	f	11	c	"+++"
Inlet Produced Water Tank 1	5/17/92	107	5.00	c	14	c	34	d	"++"
Inlet, Produced Water Tank 2	5/17/92	89	3.90	c	16	c	41	d	"++"
Inlet, Produced Water Tank 3	5/17/92	93	11.90	f	82	f	8	b	"++"
Inlet, Seawater Tank 3	5/17/92	92	2.80	b	0	a	0	a	ND
Pigging Water from Seawater line from Processing Center to Producing Area	5/4/92	97	0.70	a	4	a	4	a	ND

LEGEND:

ND	*None Detectable*
"+"	*<5 Faint Bands*
"++"	*6-10 Bands, Some Faint, Some Intense*
"+++"	*>10 Bands, Some Faint, Some More Intense*
"1"	*Corrosion Coupon Weight Loss*
"2"	*Deepest Pit in Main Body of Coupon*
"3"	*Deepest Pit at End of Coupon*
a-b	*Acceptable*
c-d	*Status is of Concern*
f	*Fails to meet Standards*
MPY	*Mils Per Year*